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Phenytoin enhances collagenization in excision wounds and tensile strength in incision wounds

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Background: Hard-to-heal wounds present a major medical problem. The anticonvulsant drug phenytoin has been shown to have prohealing effects in various types of wounds. In this study we evaluated the effect of phenytoin on some phases of wound healing in a rat excision wound model. *Methods:* A total of 98 adult male Wistar rats were used in this study. The effect of phenytoin ointment on the time for complete wound closure, as well as its biochemical and histological effects were evaluated in an excision wound. In addition, mechanical effect of phenytoin was evaluated in an incision wound rat model. *Results:* Phenytoin hastened the healing and increased protein and hydroxyproline contents as well as histological collagenization of excision wounds. In addition, it increased the tensile strength in incision wound model. *Conclusion:* This study is the first to profile in detail the effects of phenytoin on morphology and biochemistry of excision wounds. We have shown that phenytoin not only shortens the time for wound healing but also improves the quality of the healing tissue. These effects are sought for in various clinical settings in which unaided healing is inconveniently prolonged or where the forming scar is not fully developed, allowing relapse of the wound.

1. Introduction

Wounds are a very common medical problem and present a staggering economic burden (Meier and Nanney 2006; Ramsey et al. 1999; Woodbury and Houghton 2004). For example, the average total cost per patient to heal pressure ulcers within a complex care facility was estimated to be $11,084 over an average of 192 days (Woodbury and Houghton 2005). The wide spread of other chronic wounds, e.g., diabetic wounds, war-related missile wounds and venous stasis ulcers, further underscores the need for efficacious wound healing interventions.

Natural wound healing includes several overlapping stages. The first stage is inflammation, which involves the release of cytokines that initiate the next stage of proliferation. In proliferation, three processes take place: re-epithelization, formation of extracellular collagen bed and angiogenesis. Re-epithelialisation requires migration and mitosis of mesenchymal and epithelial cells; both processes rely on the production of proteoglycans and glucosamine (McCarty 1996). In the final stage of remodeling, collagen is cross-linked and aligned to increase tensile strength at the wound site leading eventually to scar formation (www.medicaledu.com/phases.htm).

The anticonvulsant drug phenytoin has been evaluated as a prohealing agent for the treatment of chronic skin ulcers (Pendse et al. 1993; Ashima and Surya 2004; Rhodes et al. 2001; Muthukumarasamy et al. 1991; Carneiro and Nyawawa 2003), large abscess cavities (Lodha et al. 1991) and burns (Ashima and Surya 2004; Carneiro et al. 2002). A recent systematic review of randomized controlled clinical trials suggested that phenytoin illustrates positive effects on wound healing in a variety of wounds (Shaw 2007). The mechanism seems to be complex since phenytoin was shown to increase the expression of about 1500 genes in human dermal fibroblasts (Swamy 2004).

Several topical dosage forms of phenytoin have been evaluated, including phenytoin powder or phenytoin sodium powder (Carneiro and Nyawawa 2003; Carneiro et al. 2002; Modaghegh et al. 1989; Vo 2001, Chauhan et al. 2003, solution (Helmke 2004), suspension (Vo 2001), cream (Vo 2001) and gel (Helmke 2004).

Prohealing agents probably exert their effects by: (i) Re-epithelization, as determined by the rate of wound contraction and the time for complete wound closure, (ii) collagenization, as reflected by biochemical analysis (protein and hydroxyproline contents within healing wounds), histological evaluation and mechanical tests, and (iii) angiogenesis, as determined by histological evaluation. Topical phenytoin has been shown to improve these parameters in incision wounds (DaCosta 1998) and large abscess cavity models (i.e. dead space) (Lodha et al. 1991).

However, the lack of detailed histological and biochemical assessment of the prohealing performance of phenytoin on excision wounds prompted us to investigate effects of this agent on large excision wounds and to profile the resulting physical and biochemical outcomes employing a rat model.

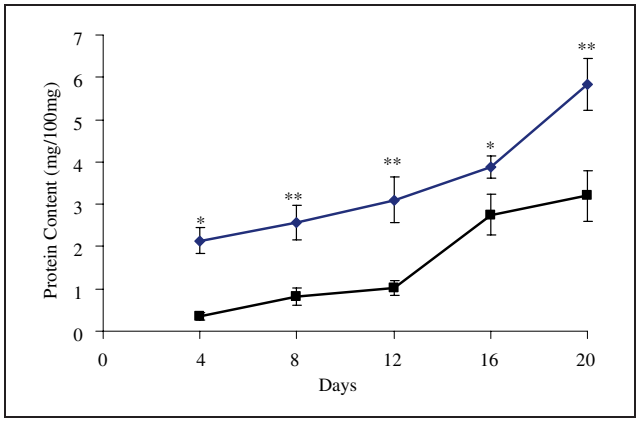

Fig. 1: Protein content (mg/100 mg granulation tissue) of wounds treated with phenytoin/vaseline ointment (◆) compared to vaseline alone (■) plotted against time (days) after induction of excision wound (at day zero). N = 5–6 for each group. * $p < 0.05$, ** $p < 0.01$

2. Investigations and results

Phenytoin illustrated significant prohealing effects on excision wounds as it significantly shortened time of complete wound closure from an average of 26.00 days (±1.32) in the case of vehicle-treated controls to 21.17 days (±1.01, $p < 0.01$). The experimental and control groups contained six rats each. Time for complete wound closure was visually estimated for each rat and compared for the two groups.

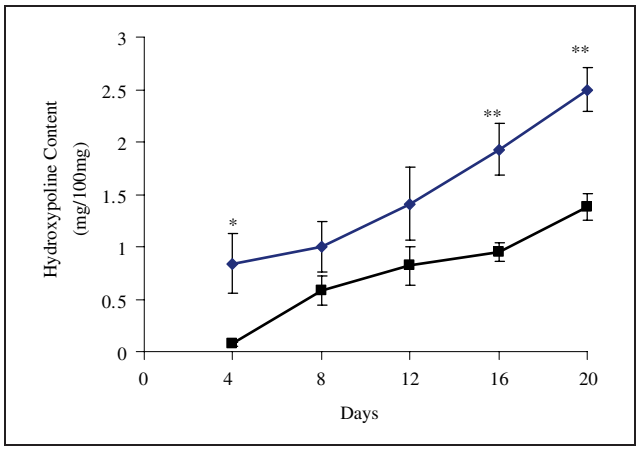

Fig. 2: Hydroxyproline contents (mg/100 mg granulation tissue) of wounds treated with phenytoin/vaseline ointment (◆) compared to vaseline alone (■) plotted against time (days) after induction of excision wound (at day zero). N = 5–6 for each group. * $p < 0.05$, ** $p < 0.01$

The prohealing effects of phenytoin are further emphasized by the significant increase in wound protein and hydroxyproline contents measured at different time intervals, as shown in Figs. 1 and 2. All data are given as mean ± SEM (standard error of the mean). 2-Sample one-tailed t-test was used to test differences between the groups. The differences were considered significant at $P < 0.05$. Similarly, phenytoin improved the deposition, arrangement and maturity of collagen fibers within the granulation tissue (Fig. 3).

Unsurprisingly, topical phenytoin accelerated healing of incision wounds: on the 10th day after incision, 4 rats from the phenytoin-treated group completed healing compared to two rats from the control group. Moreover, phenytoin improved the tensile strength of incision wounds as illustrated in Fig. 4.

3. Discussion

This study was conducted to elucidate the effect of phenytoin on different phases of healing of excision wounds. We have shown that phenytoin increases protein and hydroxyproline contents and collagenization of healing excision wounds; effects that enhance the quality of the newly formed tissue as compared with the vehicle-treated rats. Our findings are in accordance with a previous study showing that phenytoin inhibited collagenase activity of fibroblasts (Genever et al. 1995).

In addition, our findings have significant clinical implications. For example, the increased tensile strength observed with phenytoin treatment means that the newly-formed tissue is more resistant for breakage when exposed to tensile force. This property is sought for in clinical settings where incision is induced deliberately as in most invasive operations. Impaired healing of the surgical incision represents a common complication in these settings (Santangelo et al. 2006).

Since phenytoin-induced increase in protein and hydroxyproline contents (Figs. 1 and 2) correlated with the increase in wound tensile strength (Fig. 4), we postulate that the latter is related to enhancement of collagen deposition and maturity. In fact, phenytoin-induced reduction of wound closure time can also be attributed to phenytoin-enhanced collagenization (Fig. 3). It is readily observable from Fig. 3 that phenytoin accelerated wound healing so that the histology of a vaseline-treated wound at day 16 would compare to that of a phenytoin-treated wound on a much earlier day.

In conclusion, the present study supports the role of phenytoin in healing excision wounds and triggers further investigations towards the discovery of other agents of similar prohealing effects.

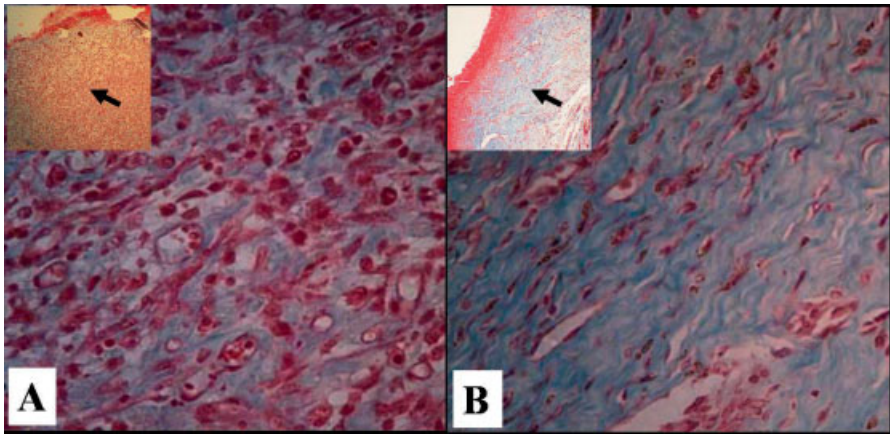

Fig. 3:
Deposition of regular collagen fibers (bluish regions) in excision wounds treated with (**A**) Vaseline control, (**B**) phenytoin. The samples were collected at the 16th day after excision. Notice the thickness, maturity, and regularity of collagen fibers in the phenytoin-treated wound tissue compared to vaseline control. (Masson Trichrome, 900 ×, inlets are 100 ×)

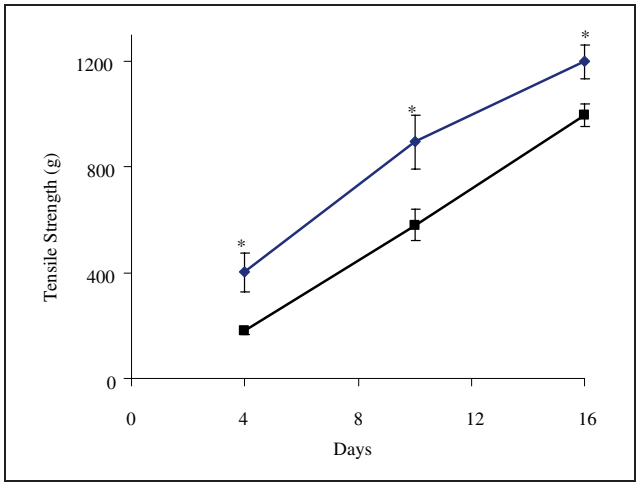

Fig. 4: Tensile strength (g) of wounds treated with phenytoin/vaseline ointment (◆) compared to vaseline alone (■) plotted against time (days) after induction of incision wound (at day zero). N = 5 for each group. * $p < 0.05$

4. Experimental

4.1. Animals

A total of 98 adult male Wistar rats (150–180 g) were used in this study. The animals were provided by animal house unit of the Department of Biological Sciences at the University of Jordan. They were provided with food and water *ad libitum* during the whole period of the experiments. The procedures involving animals and their care conformed to the international guidelines, Principles of Laboratory Animals Care.

4.2. Wound induction

4.2.1. Excision wound

Animals were anesthetized by intraperitoneal injection of 3.5% chloralhydrate at a dose of 0.35 mg/g body weight. The dorsal backs of the rats were shaved and disinfected with ethanol. Then excision wound was created by removing 2 cm × 2 cm full thickness piece of the skin from a predetermined shaved area. No local or systemic anti-microbial agents were used. The rats were distributed randomly to treatment and control groups and each rat was placed in a separate cage. Each experimental group consisted of 6 rats unless otherwise mentioned. The excision wound model was used to monitor all the parameters mentioned in the introduction except the tensile strength, which was evaluated employing an incision wound model.

4.2.2. Incision wound

Animals were anesthetized and shaved in the same way as for excision wound. However, in this case, 3 cm linear incisions to the depth of the subcutaneous tissue were made on the back skin using a blade. The two edges of the wound were then kept close together with a single suture in the middle of the wound using a surgical thread.

4.3. Ointment preparation and application

A 10% phenytoin in vaseline ointment was prepared and applied once daily over six consecutive days in all experiments. Inclusion of the wound edges was ensured. Control animals were treated similarly, but with vehicle (vaseline) alone.

4.4. Determination of protein and hydroxyproline contents

For each of the treated and control groups, excision wounds were induced in 30 rats, then six rats from each group were sacrificed at 4 day intervals up to 20 days of wounding. For each rat, the granulation tissue forming in the wound was removed, weighed and homogenized in 3 ml 0.1 M phosphate buffer (pH 7.2) using a homogenizer. The homogenate was then divided into two parts for determination of protein content using Lowry's method (Lowry et al. 1951) and hydroxyproline content using the method described by Woessner (1961).

4.5. Histological evaluation

Five micrometer-thick sections were prepared from the granulation tissue of the excision wounds in two sacrificed rats of the treated and control groups on days 4, 8, 12, and 16. The sections were stained with Masson's trichrome for the assessment of collagen content and maturation.

4.6. Determination of tensile strength

The tensile strengths of healing incision wounds were determined for treated and control groups (5 rats each) on days 4, 10 and 16 after incision. The rats were sacrificed and a rectangular section (a length of 3.4 cm and a width of 3 cm) of the skin, including the healing incision wound, excised. The tensile strengths of these sections were measured using a tensiometer designed according to the method of Vaisberg et al. (1989). In brief, one edge of the rectangle parallel to the wound was fixed while applying incremental loads to the other edge. The tensile strength was then taken to be the load in grams required to disrupt the wound.

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